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Redistribution of membrane 5′-nucleotidase in rabbit peritoneal polymorphonuclear leucocytes during phagocytosis

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Using a quantitative phagocytic model involving oil droplet internalisation rabbit polymorphonuclear leucocytes display selective segregation of membrane constituents during the phagocytic event. A resting cell surface membrane and fractions representing vesicle membranes and uninvolved surface membranes from the active cells have been purified by density gradient sedimentation and free flow electrophoresis. The specific activity of 5′-nucleotidase, a major neutrophil surface membrane glycoprotein, was 3-fold higher in the univolved membrane of phagocytosing cells than in the resting cell membrane. The activity in the vesicle membranes was substantially depleted. In contrast *Lens culinaris* receptors showed no redistribution during phagocytosis: the two surface domains showing essentially the same enrichment with respect to homogenate as the resting cell surface membrane.

Phagocytosis *5′-Nucleotidase* *Redistribution* *Surface membrane domain*

1. INTRODUCTION

Since the fluid mosaic model for membrane architecture was first proposed [1], the question of whether cell surface constituents selectively segregate into or out of specialised domains during motile events has been the subject of considerable debate. With lymphocytes ligand-induced and spontaneous expression of membrane heterogeneity and the migration of lectin and antibody–receptor complexes into patches, caps or protuberances is a well-established phenomenon [2,3]. How such lateral movements are effected is unclear but from investigations using microtubule poisons and microfilament destabilizing agents on a variety of cells there seems little doubt that cytoskeletal elements are in some way involved in these membrane segregatory and migratory processes [4–6].

In phagocytosing cells such as the polymorphonuclear (PMN) leucocyte and macrophage the evidence for segregation of membrane constituents linked to the phagocytic event is somewhat conflicting. On the one hand it has been reported [7,8] that the K_m and V_{max} of the surface transport sites for purines and amino acids were unaltered in the phagocytosing macrophage even though as much as 30–50% of the surface membrane had been internalised. The conclusion was that transport sites for these substances migrated out of forming phagocytic vesicle into uninvolved surface membrane domains. These views have been supported for other cellular phenomena in [3] where at least 15 separate determinants in the membranes of lymphocytes became heterogeneously distributed after lectin or antibody binding, and in [10] where the migration of Fc receptors in human leucocytes was followed during chemotaxis. In [11], using semi-quantitative histochemical procedures the redistribution of the ecto-enzyme 5′-nucleotidase was followed during PMN-leucocyte phagocytosis. The enzyme activity which was evenly distributed on the surface of resting leucocytes became virtually excluded from the phagosomal membrane after 5 min ingestion of bacteria. Similar experiments by the same group in which the 5′-nucleotidase activity in latex-containing phagosomes was measured biochemically confirmed this redistribution since

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after 60 min into phagocytosis the phagosomes showed <10% of the total membrane 5′-nucleotidase activity [11]. However, in a study of the distribution of lactoperoxidase-iodinated polypeptides during endocytosis, the iodinated constituents on the interior face of phagolysosomal membranes were found essentially identical to those available for iodination on the exterior face of the surface membrane [12]. Evidence that the nature of the particle engulfed may influence the degree of selective segregation was provided in [13] where antibody opsonised liposomes were compared with unmodified latex beads as the phagocytic stimuli. In the former case the expression of C3b complement receptors remained well expressed on the surface membrane but Fc receptors were significantly depleted in number. Following internalisation of latex beads, however, both types of receptor disappeared from the surface membrane.

Here, we have followed biochemically the changes in distribution of 5′-nucleotidase during phagocytosis of opsonised oil red 'O' particles by rabbit peritoneal PMN-leucocytes. With this model system, phagocytosis can be monitored spectrophotometrically and it is also possible, using gradient centrifugation, to harvest the lower density oil rich phagosomes independently of surface membrane domains uninvolved in the phagocytic process.

Our findings clearly suggest that 5′-nucleotidase, which is a major surface glycoprotein in rabbit PMN-leucocytes, is excluded out of the forming phagocytic vesicle during particle internalisation and becomes significantly enriched in specific activity with respect to resting cell surface membrane levels in the regions of the membranes not involved in vesicle formation.

2. EXPERIMENTAL

2.1. *PMN leucocyte isolation*

Polymorphonuclear leucocytes were harvested from the peritoneal cavities of rabbits following elicitations by injection into the peritoneum of 500 ml sterile saline containing 0.1% (w/v) glycogen in 0.154 M saline [14]. Withdrawal of the cells between 8–10 h after injection gave a preparation that was 96% viable and >95% PMN leucocytes with a small proportion (<3%) of lymphocytes.

2.2. *The phagocytic model*

The procedure we have used for monitoring phagocytosis was described in [15] and involves presenting to the cells a sonicated emulsion of paraffin oil into which oil red 'O' has been incorporated. The particle mixture is opsonised and stabilised with albumin. In our experiments the cells take up 3 or 4 large oil droplets and 1 or 2 smaller ones after 20 min phagocytosis. After drying down a known aliquot of cells in small glass vessels for 2 h at 80°C the dry material is dissolved in 1 ml dioxane and the absorbance measured at 524 nm.

2.3. *Membrane isolation*

The technique for washing the cells free of glycogen, of labeling at the whole cell level with ^{125}I-labelled *Lens culinaris*, and the homogenisation and isolation of a mixed membrane fraction on a sorbitol density gradient has been described in [16]. The further purification of this mixed membrane fraction is achieved using high-voltage, free-flow electrophoresis in a flowing buffer film. This procedure has been described for purifying human platelet surface membranes [17].

2.4. *Analytical procedures and enzyme assays*

Protein was generally determined as in [18] but if samples contained amounts of sorbitol which interfered, a microtannin turbidometric method was used [19]. Standard curves were prepared from bovine serum albumin. The assay of 5′-nucleotidase was carried out in the presence of 1 mM *p*-nitrophenyl phosphate with sufficient Mg^{2+} added to overcome any effects of residual EDTA from the sorbitol gradient procedure. In other respects, the assay was essentially as in [20] and involved the measurement of released adenosine from [^{3}H]AMP after its precipitation with $Ba(OH)_2$ and $ZnSO_4$. A final concentration of 60–80 mM $Ba(OH)_2$ was found to be optimum for a 200 μl aliquot containing the enzyme reaction products. With $\leqslant$100 μg membrane protein the assay was linear up to 40 min incubation. An antibody to rat liver membrane 5′-nucleotidase which was used to confirm the presence and disposition of leucocyte membrane 5′-nucleotidase

had no effect upon phosphodiesterase, nucleotide pyrophosphatase $Mg^{2+}[Na^+,K^+]$-ATPase or the non-specific phosphatase inhibited with *p*-nitrophenyl phosphate. This antibody was kindly provided by Dr H. Evans of the MRC Laboratories (Mill Hill). The iodination of *Lens culinaris* was done following [21]. The leucocytes were labelled with this lectin at the whole cell level as in [22]. There was no demonstrable effect of this label on either cell viability or their capacity to phagocytose in these studies.

3. RESULTS AND DISCUSSION

Fig.1 shows a typical curve for phagocytosis of oil red O particles by the PMN-leucocytes measured by uptake of the dye into the cells using spectrophotometric analysis after extraction with dioxane. Although the process is a little slower than with human granulocytes the rabbit cells reach maximum at ~10 min and the plateau is maintained for at least a further 20 min. One must presume that cells at plateau are reluctant to compromise their surface membrane in further phagocytosing presented particles. In the following experiments, cells were taken for processing after 15–20 min into phagocytosis.

Fig.2 shows the sorbitol density gradient appearance after the separation of homogenates prepared from resting and phagocytosing PMN-leucocytes. In the case of the phagocytosing cells the fraction which accounted for all the oil red 'O' in the homogenate and representing phagocytic

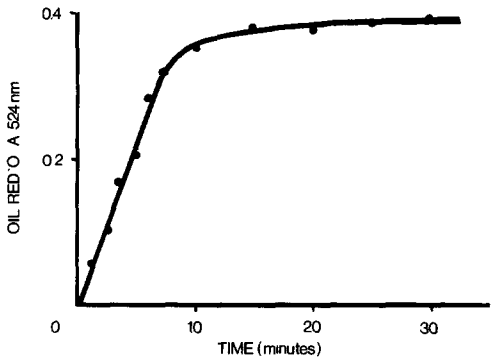

Fig.1. Time curve for the phagocytosis of opsonised paraffin oil droplets in which the dye oil red 'O' has been incorporated. The spectrophotometric analysis of the internalised droplets is described in the text.

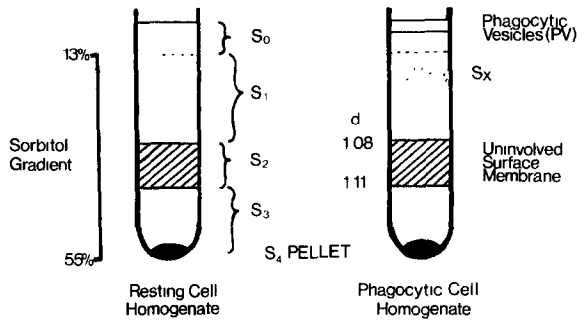

Fig.2. Diagrams of the appearance of the sorbitol density gradients after separation of homogenates from resting and phagocytosing PMN-leucocytes. The membrane vesicles locating between 1.08–1.11 density represent mixed membrane fractions containing vesicles of surface and intracellular origin. These are further purified by free flow electrophoresis. The phagocytic vesicles locate close to the meniscus in gradients of homogenates from phagocytosing leucocytes.

vesicles locates close to the meniscus in the gradient and was collected independently of the lower membrane fraction which represents membrane elements not involved in the internalisation of the oil droplets. Occasionally in the density gradient separations of homogenates from phagocytosing cells a very faint but discrete particulate band (S_x, fig.2) was visible between the phagocytic vesicle zone and the surface membrane zone. The protein content of this band was < 15% of the amount present in the membrane zone. By pooling this material from a number of gradients sufficient was obtained for one SDS–polyacrylamide gel electrophoresis separation. The polypeptide profile was clearly different from that of the surface membrane but the low yield and infrequency of appearance precluded any further characterisation of this fraction. Transmission electron micrography of phagocytic vesicles taken from the low density meniscus zone of the gradient from phagocytosing cells showed oil droplets surrounded by a boundary membrane. This membrane was freed from the included oil by hypotonic lysis [20 mM Tris–HCl (pH 7.4)] for 1–2 min and the membrane deposited by high speed centrifugation ($6 \times 10^6 \times g$ min). This high speed pellet was uncontaminated by dye after washing once with isotonic saline and recentrifugation. Fig.3a,b show the free flow electrophoresis profiles after applying the mixed membrane fraction from resting and

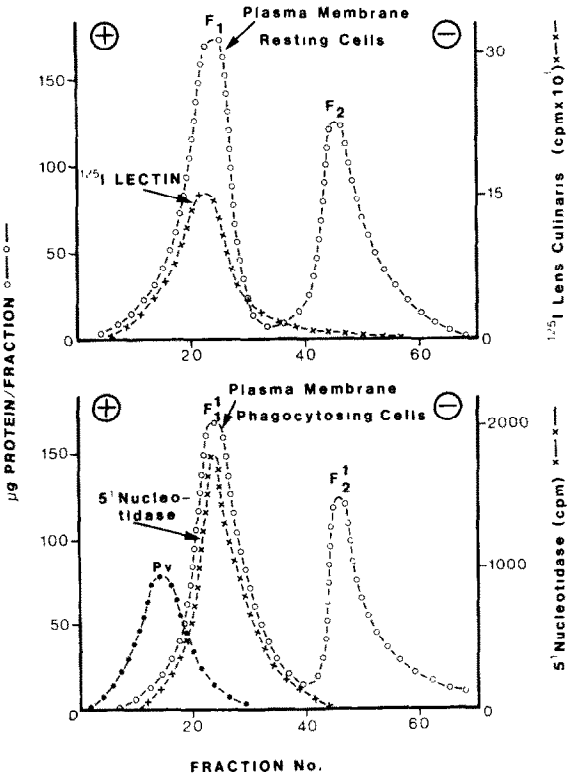

Fig.3. Composite diagram of the free flow-electrophoresis profiles from resting (a) and phagocytosing (b) cells. The F_1 and F_2^1 fractions represent surface membranes as indicated by almost exclusive localisation of the ^{125}I-labelled *Lens culinaris* and 5′-nucleotidase activity. Their electrophoretic mobilities are identical. The peak 'Pv' in the lower profile represents phagocytic vesicle membranes. These are normally electrophoresed separately but the profile is superimposed here for comparison.

phagocytosing cells to the chamber of the apparatus. The control and experimental cell membrane fractions have almost exactly the same electrophoretic mobility. Fractions across the peaks usually within the limits of the width of the peak at half height and containing the 5′-nucleotidase activity and [^{125}I]lectin were pooled for analytical purposes. Superimposed upon the lower electrophoretic diagram 4b is the profile of the phagocytic vesicles electrophoresed independently of the mixed membrane fraction.

In routine experiments the purified surface membrane fraction isolated from resting cells was 18 ± 0.8- and 6.2 ± 0.9-fold enriched with respect

Table 1

Redistribution of [^{125}I]lectin and 5′-nucleotidase activity

[^{125}I]Lectin (dpm × 10^3 mg protein^{-1})	Resting cells	Phagocytosing cells
Homogenate	244 (1.0)	222 (1.0)
Surface membrane	2070 (8.5)	1980 (8.9)
Phagocytic vesicle membrane	–	1830 (8.3)
5′-Nucleotidase activity (nmol AMP.mg protein^{-1}.h^{-1})		
Homogenate	1.2 (1.0)	1.3 (1.0)
Surface membrane	21.6 (18.0)	45.0 (34.5)
Phagocytic vesicle membrane	–	1.4 (1.0)

Figures in parentheses are enrichments of the specific activities with respect to homogenate values = 1.0

to 5′-nucleotidase activity and ^{125}I-labelled *Lens culinaris* when compared with the activity in the starting cell homogenate. Table 1 shows the results from a typical experiment in which the values for 5′-nucleotidase activity and bound ^{125}I-labelled *Lens culinaris* have been directly compared in resting and phagocytosing cells taken from the same rabbit. It will be seen that *Lens culinaris* receptors do not redistribute during phagocytosis showing approximately the same enrichment with respect to homogenate counts for [^{125}I]lectin in the surface membrane of the resting cells, the uninvolved membrane of the phagocytic cells as well as in the membrane of the phagocytic vesicles analysed independently. In contrast, however, 5′-nucleotidase-specific activities which are 18-fold enriched with respect to homogenate in the resting cells are around 35-fold enriched in the uninvolved surface membrane of the phagocytosing cells and the specific activity depleted to homogenate levels in the membrane of the phagocytic vesicles. This implies that a considerable redistribution has occurred during the phagocytic event with the enzyme being selectively excluded out of the phagocytic vesicle domain.

Table 2 shows the results from 3 successive experiments in which the activity of 5′-nucleotidase

Table 2

Selective segregation of 5′-nucleotidase activity within the membrane during phagocytosis

Cell fractions		5′-Nucleotidase activity (nmol AMP.mg protein^{-1}.h^{-1})	
		Resting cells	Phagocytosing cells
Homogenate		1.2	1.0
Surface membrane preparations	1	7.7	36.0 (4.4)
	2	14.6	42.0 (2.9)
	3	16.7	49.0 (2.9)
	Mean	13.0	45.0 (3.4)
Phagocytic vesicle membranes	1	–	1.8
	2	–	1.4
	3	–	1.7
		Mean	1.6

Values in parentheses represent enrichments of the specific activities with respect to activities in the corresponding resting cell surface membranes

was similarly compared in the various membrane fractions. On a mean basis the 5′-nucleotidase-specific activity in the subfraction representing the uninvolved surface membrane of the phagocytosing cells is >3-times higher than the specific activity in the whole surface membrane of resting leucocytes. Again the phagocytic vesicle membrane activity expressed with respect to membrane protein approaches the specific activity levels found in the whole homogenate thus again supporting a very substantial segregation out of the vesicle membrane into the uninvolved surface domain.

These enrichment values may in fact be considerably higher in real terms, since from a parallel study (to be reported elsewhere) in which the dynamic aspects and distribution of the intracellular contractile protein actin has been investigated there is a substantial increase in filamentous actin (about 40%) associated with the uninvolved surface membrane during phagocytosis. Since SDS–polyacrylamide gel electrophoresis of the polypeptide profiles of the surface membranes from resting and phagocytosing cells showed no detectable differences other than a substantial increase in the 43 kDa actin band, the enrichment figures for the specific activity of 5′-nucleotidase in the uninvolved surface membrane of the phagocytic cell may be artefactually low because of the assembly of actin on its cytoplasmic face.

In confirmation that we have been measuring a membrane constituent with surface oriented enzyme activity the surface membrane vesicles from resting leucocytes (which always appeared to be sealed in electron micrographs) were treated with the anti-5′-nucleotidase antibody.

Fig.4 shows a typical inhibition curve in which at maximum the enzyme is 83% inhibited by this antibody. The assay was performed in the presence of excess *p*-nitrophenyl phosphate. Total inhibition of the activity could not generally be achieved because at high concentrations the antibody reduced the efficiency of the $Ba(OH)_2$ precipitation of the unreacted [^{3}H]AMP in the enzyme assay procedure.

In conclusion, we believe that in this experimental phagocytosis system, which is perhaps a 'frustrated phagocytic' model since it does not progress to the formation of phagolysosomes and granule secretion, we have provided strong evidence for the selective segregation of 5′-nucleotidase out of the forming phagocytic vesicles. Its selectivity is also reflected in the failure to demonstrate any change in the distribution of *Lens culinaris* receptors into or out of the phagocytic vesicles during the phagocytic process.

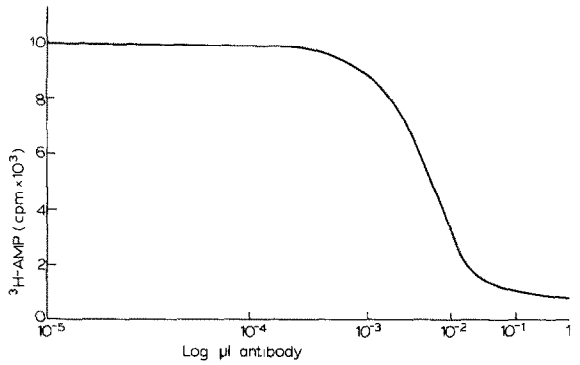

Fig.4. Samples (20 µg) of the leucocyte surface membrane were incubated for 30 min at 37°C in the presence of increasing concentrations of anti-5′-nucleotidase antibody and the 5′-nucleotidase activity measured as in [7] using [^{3}H]AMP.

Our studies therefore confirm the histochemical redistribution of this enzyme during phagocytosis as shown in [11] using guinea pig peritoneal PMN leucocytes. These authors reported an almost total clearance of 5′-nucleotidase activity from the phagosomal membranes following latex particle internalisation. This was in clear contrast to its relatively even distribution over the surface membrane of resting cells.

However, our studies conflict with [23], in which the more random distribution of 5′-nucleotidase in rabbit neutrophil membranes persisted after phagocytosis with little evidence of any selective segregation. It is possible that in some membrane isolation procedures one may be selectively isolating 'hot spots' or membrane domains that are not representative, and although we cannot totally exclude this in our own experiments we feel that by the combination of density gradient sedimentation and further purification by free flow electrophoresis, together with separate analyses of the phagocytic vesicle membranes, we should have ensured less contamination of the fractions than has been possible. This we believe is reflected in our high enrichment values for various surface markers. Moreover, by the comparison of the distribution of *Lens culinaris* receptors which do not appear to migrate laterally concomitant with the changes in the disposition of 5′-nucleotidase, we would support the view that there are dynamic processes of high selectivity proceeding within the membrane lipid bilayer during phagocytic events. The phagocytic model and the membrane isolation procedures described here are highly suitable for the study of other membrane constituents and receptors which may or may not selectively segregate during particle internalisation.

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